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Generation of genome-modified *Drosophila* cell lines using SwAP

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Abstract

The ease of generating genetically modified animals and cell lines has been markedly increased by the recent development of the versatile CRISPR/Cas9 tool. However, while the isolation of isogenic cell populations is usually straightforward for mammalian cell lines, the generation of clonal *Drosophila* cell lines has remained a longstanding challenge, hampered by the difficulty of getting *Drosophila* cells to grow at low densities. Here, we describe a highly efficient workflow to generate clonal Cas9-engineered *Drosophila* cell lines using a combination of cell pools, limiting dilution in conditioned medium and PCR with allele-specific primers, enabling the efficient selection of a clonal cell line with a suitable mutation profile. We validate the protocol by documenting the isolation, selection and verification of eight independently Cas9-edited *armadillo* mutant *Drosophila* cell lines. Our method provides a powerful and simple workflow that improves the utility of *Drosophila* cells for genetic studies with CRISPR/Cas9.

Introduction

The discovery and adaptation of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system and its application in diverse species, including yeast,[1] fruit fly,[2-7] zebrafish,[8-10] mouse,[11-13] and human cells [14,15] has reshaped the landscape of molecular biology. Today, scientists are able to easily and efficiently engineer virtually any genome at specific loci.[16-18] The Cas9 protein is an RNA-guided DNA endonuclease recognizing a short trinucleotide NGG protospacer motif sequence (PAM) adjacent to the cognate target sequence.[19,20] Subsequent Cas9 cleavage of the double-stranded DNA is followed by DNA repair events. The introduced double-strand breaks are mended either by non-homologous end joining (NHEJ) potentially leading to mutational events in the target site or by homology directed repair via a donor template.[21,22]

The CRISPR/Cas9 system has also been implemented in cultured *Drosophila* cell lines.[23,24] *Drosophila* cell lines are an widely used experimental system, complementing the insights into basic biological mechanisms, genes functions, and disease obtained in flies (for a review, see ref. 25). The advantages of fly cell culture over mammalian cells are of technical and biological nature, such as their high susceptibility to RNAi and their simple genomic structure with less redundancy, providing a powerful gene discovery tool.[26,27] Currently, more than 150 fly cell lines are publicly available from the Drosophila Genomics Resource Center (DGRC) among which, S2, Clone-8 and Kc167 are the most commonly used ones. These lines have also been used for large-scale studies such as the modENCODE project investigating genomic structural elements.[28]

The applications of the CRISPR/Cas9 system in *Drosophila* tissue culture ranges from its general applications of generating genetic mutations,[23,24,29] to CRISPR interference studies [30,31] and the establishment of a genome-wide CRISPR library for high-throughput screens.[32] However, one historical challenge when working with *Drosophila* cells is their difficulty to grow at low densities probably as they require

essential growth stimulating factors secreted from neighboring cells.[33] This problem impedes the generation of clonal genetically modified *Drosophila* cell lines vitiating potential advantages gained by implementing targeted genome editing technologies such as CRISPR/Cas9. Several methods for cloning have been reported such as cloning by limiting dilution in conditioned medium,[34] irradiated feeder layer cells or soft agar plates (for a review, see ref. 33). However these methods are not widely used because of the low cloning efficiencies and the significant amount of time and work needed to isolate clonal lines, especially when no selectable markers (e. g. fluorescence, drug resistance) are used to isolate the clone of interest. Indeed, to our knowledge in addition to our previous study, only another research article has reported the successful generation of isogenic Cas9-engineered *Drosophila* cell lines.[31,35]

Following up on our initial publication, here we describe in detail an efficient workflow that overcomes the impediments to isolating clonal, CRISPRed *Drosophila* cell lines. We have developed a selection protocol, named SwAP (pre-Selection with Allele-specific Primers) that enables *Drosophila* researchers to efficiently identify, isolate and discriminate Cas9-engineered *Drosophila* cell clones. Our method is based on combining (i) the speed and scale of cell pools to first determine pools of cells carrying a CRISPR induced modification (or combination thereof) of interest by sequencing, (ii) from this simplified population limiting dilution in conditioned medium is used for cell cloning and (iii) allele-specific (AS) primers are used to easily identify the clone of interest, which can then be expanded (Fig 1).[36-38] Using this approach, researchers can efficiently determine the allelic status and then isolate clonal cell lines with suitable mutation profiles in little time. Other common genotyping approaches used in conjunction with the CRISPR/Cas9 system, including the Surveyor assay (Cel1), T7 endonuclease 1 (for review, see ref 39), HRMA [40] and PAGE, [41] do not provide this level of detail. Here, we illustrate the general applicability of our approach by describing the generation of clonal *armadillo* (*arm*)

mutant cell lines. Our protocol does not only greatly reduce the time and work requirements for generating clonal genome-engineered *Drosophila* cells but also meets the demand for an efficient cell cloning and selection strategy in the era of CRISPR/Cas9.

Results and Discussion

CRISPRing *Drosophila* cells

Our goal was to devise a strategy that mitigates the difficult challenge of generating isogenic CRISPRed *Drosophila* cells lines. To develop such a protocol, we used *Drosophila* Kc167 cells from DGRC; they are derived from embryonic hemocytes lineages and are pseudo diploid.[42] We choose to generate additional clonal lines in which we had genomically engineered the *arm* gene.[31] We applied the same CRISPR strategy used in our recent publication to abrogate the function of *armadillo* (*arm*) with CRISPR/Cas9: two independent sgRNAs targeting the *arm* locus (Fig 2A). We transfected the cells simultaneously with two pAc-sgRNA-Cas9 expression vectors, each harboring one gRNA expression unit that targeted either the second or the third *arm* exon (Fig 2A). The vector also contains the puromycin resistance gene as a selection marker.[23] Transfected cells were selected 24 h after transfection in medium containing 5µg/ml puromycin. To avoid randomized, stable integration of the expression vector, selective pressure was stopped after 5 days.

Determination of the spectrum of genomic modifications

Having CRISPRed the cells we next wanted to identify the types of modifications that have been generated. We therefore analyzed the gRNA target sites by PCR and Sanger sequencing to determine whether the targeted locus exhibits a big deletion of 2700 bp that had been created by simultaneous cut events at both sites or if only smaller indel mutations at each individual target site had been generated. We collected an aliquot of approximately 1000 cells of our transfected cell population, extracted genomic DNA and first determined by PCR whether a big deletion has been generated; we used primers flanking the cut sites (Fig 2B). The PCR results

revealed that within our CRISPRed cell population some cells indeed harbored, at least, one *arm* allele resulting from the big deletion: an amplicon of around 550 bp was detected which would be expected if a deletion of 2700 bp had occurred. Next, we amplified each targeted site by PCR with primers spanning the individual cleavage sites to detect whether single cuts were present. To this end, we isolated the PCR products and cloned them into the pGEMT-vector (Promega). The pGEMT-system allows efficient cloning of PCR products as the linearized vector provides compatible 3´-T overhangs at the insertion site for PCR products with polymerase-added desoxyadenosine (see Material and Methods). We sequenced DNA from 10 independent colonies for each target site and were able to detect various indel mutations at the predicted cleavage locus. Analysis of the sequences revealed that 50% of sequenced alleles had indel mutations in the second exon and 57% of alleles showed mutational events at target site two (Fig 2B). As at least 50% of the alleles present were still wildtype at both target sites, we re-CRISPRed the cell population once more using the same setup to obtain a higher proportion of mutated alleles.

Isolation of pools or clumps of *Drosophila* cells carrying modified *arm* alleles

The process of *Drosophila* single cell cloning is very time and work intensive with poor cloning efficiencies. We therefore first pre-selected cell populations with cells carrying genetic modifications of interest. Suitable cell pools would then be processed further. Like *Drosophila* S2 cells, Kc167 cells will start to grow in clumps at a density greater than 10^7 cells/ml,[43] which can be easily harvested and grow quickly in comparison to single cells. We hypothesized that these cell clusters (hereafter referred to as cell pools), can be exploited to identify whether some cells of a cell pool would be favorable for single cell cloning by analyzing the mutagenic events at the *arm* locus in the respective cell pool. This step may also be of great interest for researchers that look for specific genomic variations such as frameshift

mutations. In addition, by identifying the genomic modifications we could design AS-primers to facilitate selection of the clone of interest (see below).

We picked 10 individual cell pools from our double CRISPRed cell population using a 1 µl-pipette and cultivated them in 50 µl of 50% conditioned and 50% fresh medium supplemented with 10% FBS in a 96 well plate. We harvested conditioned medium from confluent cells by removing the cells using centrifugation (see Material and Methods). After around 10 days, when the cells were about 50% confluent, we transferred the individual cell pools into a bigger well. As described above we determined the spectrum of mutations at the *arm* locus that are present in the cell pools. The results below describe the characterization of one such pool. We first established whether the isolated cell pool carries the big deletion of 2700 bp by PCR. In the first cell pool we analyzed, no big deletion was present since no corresponding PCR product could be amplified (Fig 2C). Next, we searched for the presence of mutational events at each cutting site by PCR using primers spanning the cut site and subsequent sub-cloning of the PCR products into the pGEMT-vector. We sequenced approximately 20 sub-cloned colonies for each site to gain a representative view of the genomic variation in the cell pool. The sequencing results revealed seven different types of mutations at the first target site (Fig 2C). The majority of sequences possessed either a deletion of one nucleotide (38%) or a deletion of 16 nucleotides (42%). Analysis of the sequencing results from the second target site showed that 89% of the alleles had a single nucleotide deletion (Fig 2C). Critically, no wildtype alleles were detected. As most of the detected alleles had either a deletion of one or 16 nucleotides at target site one (38% and 42%) and almost all alleles a one-nucleotide deletion at target site two (89%), we reasoned that these mutations would be suitable to select single clones for using AS-PCR (Fig 2D). We used the cells from the examined cell pool for single cell cloning. Moreover, the mutations we select for would in combination lead to a loss of Arm function.

Cell cloning

Several cloning protocols for *Drosophila* cells have been proposed (for a review, see ref 33). Best cloning efficiencies have been achieved for *Drosophila* Kc167 cells by dilution in conditioned medium.[44] Hence, we plated approximately 144 single cells to be cloned from the above described cell pool by limiting dilution in 50 µl 50% conditioned and 50% fresh medium, supplemented with 10% FBS in 96 well plates (Material and Methods).[34] After around 20 days, we could observe small cell colonies covering some of the wells. Once a clone covered half of the well, we transferred the cells to larger volumes and plates. We obtained cell cloning efficiencies of around 24%, permitting the expansion of 35 clones, of which 8 (11.5%) clones could be stably cultivated. Similar cloning efficiencies (~5%) have been reported from other laboratories.[34] As soon as cell populations were stably established we identified the clones for the desired mutations by using AS-primers (see below).

Designing allele-specific primers

Allele-specific primers allow the detection of single nucleotide polymorphism (SNP) as they have the 3'-end complementary to the SNP site.[37] We wanted to select clones possessing either the deletion of one or 16 nucleotides at the first target site and the one nucleotide deletion at the target site two at the *arm* locus (Fig 2D), we designed AS-primers based on the desired sequences according to the criteria of ref. 38 (see Material and Methods and SFig1). As described in from Liu and colleagues, we modified the three bases closest to the 3'end of the forward primer, as these are essential for primer specificity.[38] No PCR product will be generated, if at least two mismatch base pairs are present within the third bases closest to the 3'end. By contrast, a PCR product will arise, if only one mismatch occurs at the 3'end. According to the same principals, we also designed primers specific for targeting the corresponding wildtype sequences as control (see Material and Methods and SFig1).

Genotyping cells using allele-specific primers

So far many genotyping approaches have been described for genome-modified animals and human cells generated by CRISPR/Cas9, such as the commonly used

surveyor assay and HRMA (for a review, see ref. 39). Although the approaches can precisely detect, whether genomic modification events have occurred at the cleavage site, they do not provide information about the type of genomic modification. Hence, to identify and select efficiently a clonal cell line with a suitable mutations profile, we used AS-primers for genotyping. *Drosophila* Kc167 cells are pseudo diploid,[42] therefore isolated and expanded clones derived from the analyzed cell pool are expected to be either homozygous or heterozygous for the selected mutations at target site one. It is important to note that *Drosophila* cell lines may vary in their karyotype status, such as for instance S2 cells having a tetraploid karyotype.[45] In case cell line other than Kc167 are used; the selection and screening criteria need be adapted accordingly. Using AS-primer specifically detecting the deletion of one nucleotide at target site one, we observed a PCR product from all clones (Fig 3A), whereas no PCR was found from the AS-primer specific for the wildtype primer binding site (Fig 3A), indicating that all isolated clones harbor this deletion. Using AS-primers specific for the deletion of 16 nucleotides deletion we again observed a product in all clones (Fig 3B). As expected if the other allele harbored only the single nucleotide deletion and was wildtype at the primer binding site we detected a product using the respective wildtype primer (Fig 3B). At target site two the isolated clones were homozygous mutant for the single nucleotide deletion (Fig 3C). Based on the above results, we conclude that generated cell clones are heterozygous for the mutations at target site one and homozygous for mutations at target site two (Fig 3D and SFig2). Most importantly, they do not harbor any wildtype allele clearly indicating a complete loss-of function of *arm* gene function. As most widely used *Drosophila* cell lines, could carry copy number variations,[46,47] as next step targeted high-throughput sequencing methods could be applied to fully characterize the ploidy state of a generated cell clone. This step might be especially useful, when working with *Drosophila* cell lines with a non-diploid karyotype status.

Conclusion

Our paper describes a simple and efficient workflow for the generation of clonal, CRISPR/Cas9-edited *Drosophila* cell lines. The technique could essentially be used as is for most Drosophila cell lines; the parts which may need to be adapted are the method of transfection and the way in which single cell clones are isolated. Combining the speed and scale of sequenced cell pools with the effectiveness of AS-primers allows researchers to identify and select clones with a suitable mutation profile in little time. We have demonstrated the general applicability of our approach by generating 8 clonal cell lines mutant for *arm*.

Material and Methods

Cell culture and transfection

Drosophila Kc167 cells (DGRC) were grown at 25°C in M3 + BYPE medium supplemented with 5% fetal bovine serum (FBS) (Gibco) containing 1% penicillin, streptomycin (Sigma). For the transfection, $2x10^6$ cells per well in 2 ml medium in 6 well plates were seeded and transfected with a gRNA-Cas9 expression vector (pAc-sgRNA-Cas9 expression vector from Ji-Long Liu Addgene #49330) using Fugene HD (Promega) transfection reagent according to manufacturer's protocol. We used a 1:2 ratio reagent to vector with a total of 2 µg vector for each well. We recommend including transfection controls (e.g. a GFP plasmid) to monitor the transfection efficiency. Cells were selected in medium containing 5µg/ml puromycin (P8833 Sigma) for 5 days. After selection, cells were washed two times with Phosphate-buffered saline and cultivated in medium without selection marker. The region of the gene to target was determined using the tool http://www.flyrnai.org/crispr2/ and oligonucleotides were designed and cloned as described in manufacturer`s protocol.

Detection of NHEJ events by PCR and Sanger sequencing

After selection on puromycin an aliquot of approximately 1000 transfected cells were assayed for genomic modifications at the cleavage sites within the *arm* locus (FBgn0000117) (Fig 2). Genomic DNA was extracted (e.g. by using DNA-purification kit from Macherey and Nagel) and subjected for a 50 µl PCR reaction using primers

spanning over the cleavage sites (Table S1) and the GoTaq2-DNA polymerase (Promega). The GoTaq2-polymerase generates sticky ended 3' A-tailed fragments, so that PCR amplicons could be subsequently cloned into the pGEM®T Easy Vector System (Promega), which has compatible 3´-T overhangs at the insertion site. Next, we examined 10 colonies obtained by positive blue-white selection with Sanger sequencing and analyzed the sequencing results using the sequence viewer CLC Workbench.

Cloning by limiting dilution in conditioned medium

Clonal *Drosophila* cell lines were obtained according to the Linquist protocol which combines limiting dilution with the use of conditioned medium mixed with fresh medium supplemented with 10% FBS.[34] We harvested conditioned medium from confluent wildtype Kc167 cells grown over 2 days (> 10^6 cells/ml) by harvesting the cells using centrifugation at 3000 g, 5 min at room temperature. We recommend not using a cell population grown over night. We carefully removed the supernatant – the conditioned medium – and mixed it with fresh medium (1:1 ratio) and supplemented the mixture with 10 % FBS, 1% P/S. To isolate single cells limiting dilution was performed with an amount of 50 µl per well of a 96 well plate. Lids were closed with parafilm to avoid desiccation. Later that day or the next day wells were identified containing single cells. Cell clones should be identifiable after around 20 days. Once clones have covered half of the well, they were transferred to larger volumes and plates using filtered tips and used for genotyping. Due to the nature of *Drosophila* cells, we recommend to be careful by transferring cell clones to the next bigger plate and rather to wait until they cover more than half of the well.

Design of allele-specific primers for genotyping

Allele-specific primers were designed according to the concept of Liu and colleagues (see main text).[38] General rules for the PCR primer design were applied. Furthermore, we evaluated designed primers using the software AmplifX 1.5.4. by testing for the formation of hairpin loops, dimers and duplex formation.

Genotyping analysis using allele specific primers

Genomic DNA was extract from isolated and expanded cell clones using e.g. DNA extraction kit (Macherey and Nagel). We examined only a small amount of approximately 100 cells due to their sensibility to density. We analyzed the genotype of the clones using a standard PCR reaction with designed AS-primers as well as corresponding reverse primers (STable1). PCRs were performed in a total volume of 20 µl. As negative controls wildtype cells and water were used.

Abbreviations: Arm, Armadillo; AS, Allele-Specific; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DGRC, Drosophila Genomics Resource Center; NHEJ, Non-Homologous End Joining; SwAP, pre-Selection with Allele-specific Primers

Disclosure statement:

The authors have declared that no competing interests exist.

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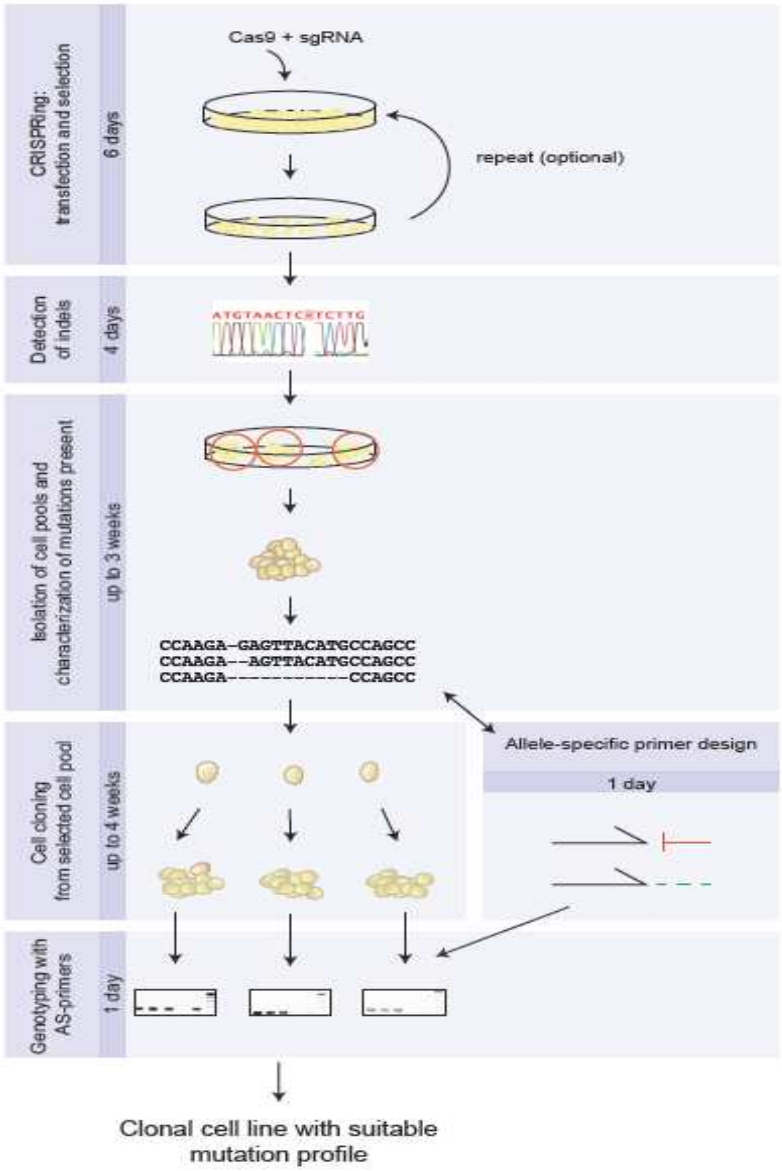

Figure 1. Overview of the workflow for the selection and identification of clonal Cas9-engineered *Drosophila* cell lines. Steps with timelines for CRISPRing cells, detection of indels using PCR and Sanger sequencing, isolation and characterization of cell pools, single cell cloning and genotyping using AS-primers and Sanger sequencing are schematically depicted. Transfected cells are selected in puromycin for 5 days and optionally reCRISPRed if a poor efficiency is observed (6 days). Selected cells are assayed for Cas9-mediated genomic modifications using PCR and sequencing (4 days). In the next step, individual cell pools are isolated and cultivated (~ 2 weeks) and their genetic modifications are examined to pre-select efficiently favorable CRISPRed cells for single cell cloning (4 days). The sequencing results are

also used to design AS-primers to screen single cell clones for desired mutation (1 day). In the next step, single cells from the selected cell pool are isolated and expanded using limiting dilution in conditioned medium (~ 4 weeks). In the last step AS-primers specifically targeting the desired mutation are used for genotyping (1 day).

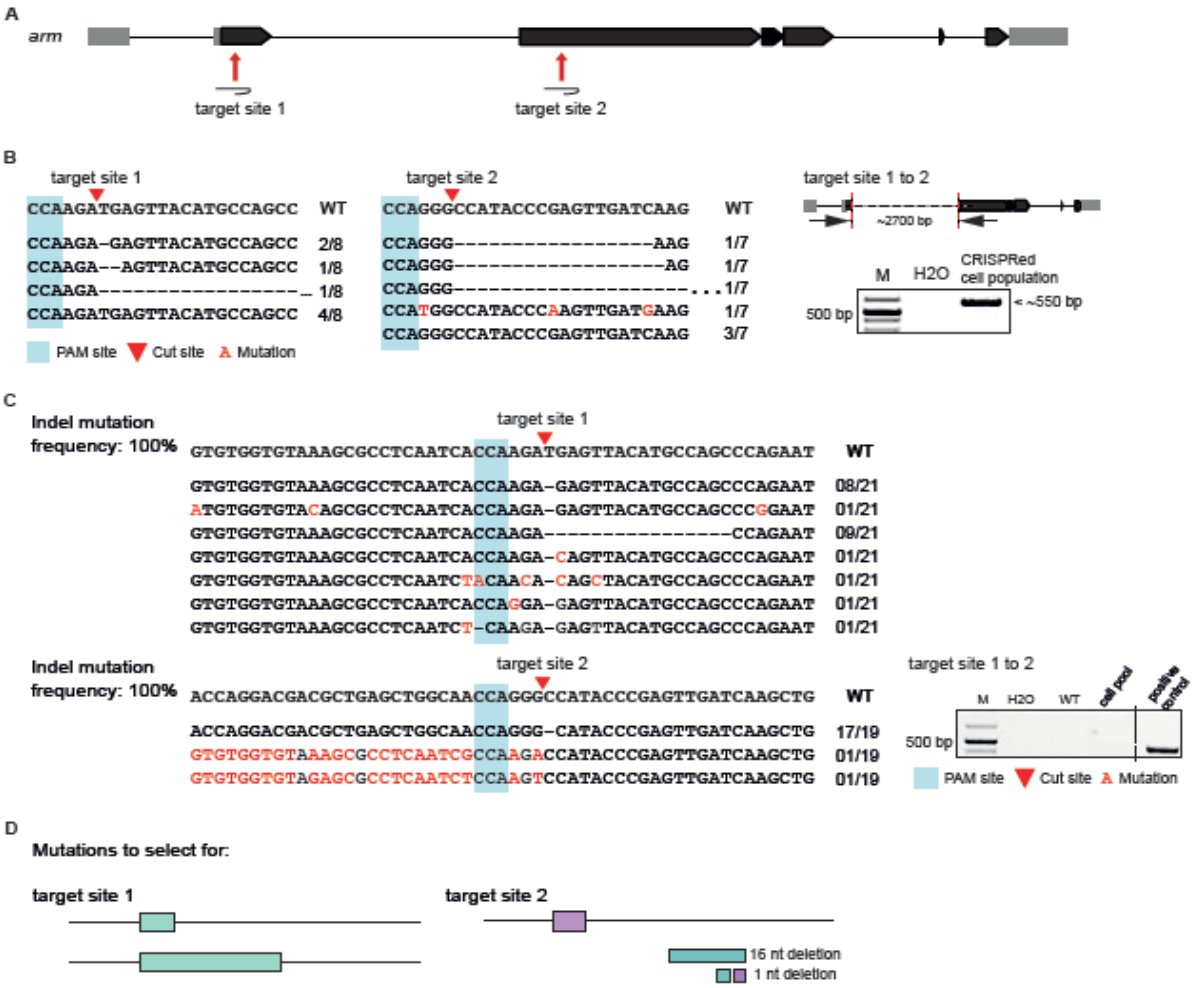

Figure 2. Detected mutations in the *armadillo* gene. (A) Schematic diagram of the *arm* locus and position of the target site 1 and 2 (red arrow). Untranslated regions (UTR) are indicated in grey boxes, translated exons in black. (B) Sequencing of indel mutations at target site one and two after transfection. PCR products spanning the cleavage site are cloned and sequenced from CRISPRed cells. The first line represents the wildtype sequence (bold). The PAM site is highlighted in blue. Schematic representation of the big deletion of 2700 bp and PCR results from primers flanking target site 1 and 2. H_2O served as control. (C) Mutational events in the analyzed cell pool. PCR produces spanning the targeted site one and two are sub-cloned and sequenced. First line represents the wildtype sequence (bold). All examined clones show indel mutations. The PAM site is highlighted in blue. PCR

results detecting the big deletion of 2700 bp from wildtype (WT), water (H_2O) and cell pool. (D) Schematic representation of mutations selected clones for.

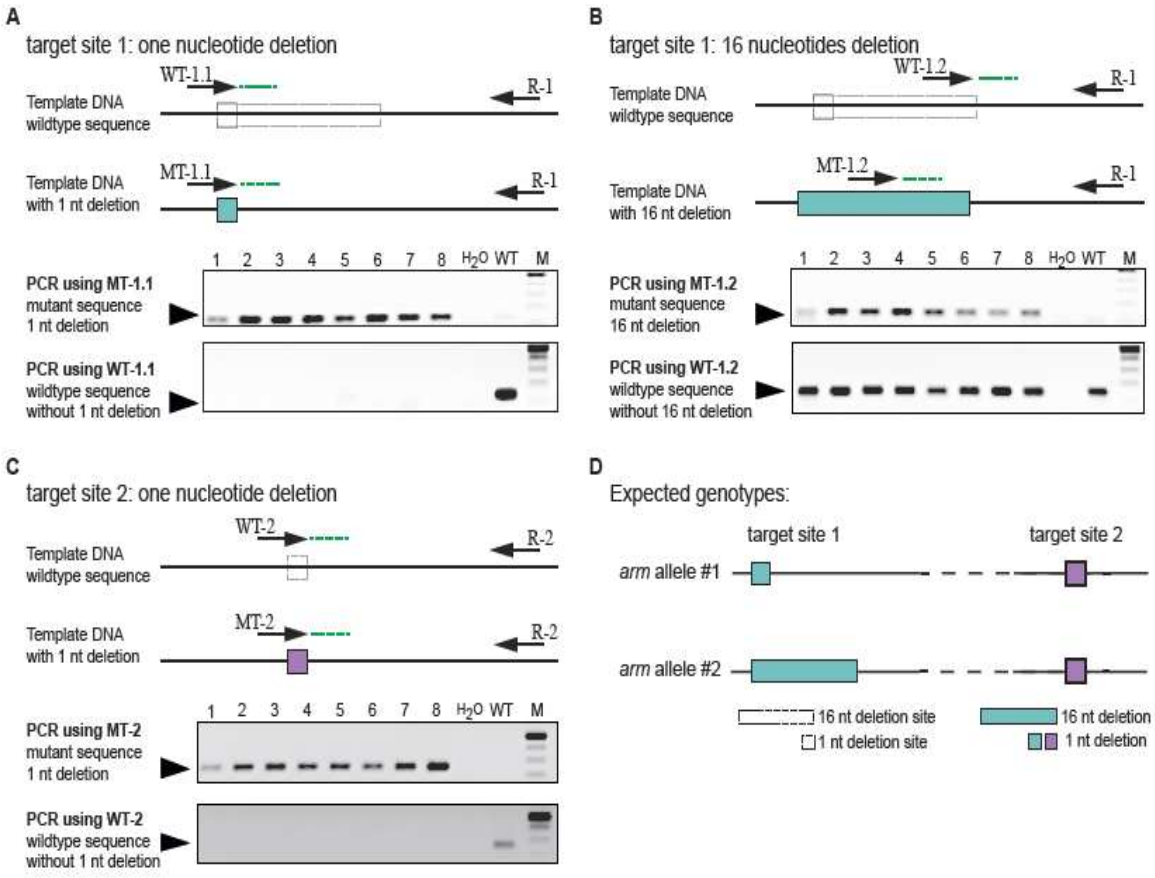

Figure 3. Schematic of AS-PCR reaction and genotyping results using AS-primers. DNA samples from eight individual *arm* mutant clonal cell lines (1-8) and from wildtype cells (WT) are genotyped using a standard PCR reaction. H_2O served as control. Arrows symbolize primers; boxes with dotted lines represent the deletion site, boxes with solid lines represent the deletion. Green dotted lines represent the ability of the AS-primer to bind. (A) AS-PCR for the deletion of one nucleotide or the corresponding wildtype sequence at target site one. PCR is performed with primers targeting one nucleotide deletion (MT-1.1) or the corresponding wildtype sequence (WT-1.1). For all PCR reactions a common reverse primer is used (R-1). (B) AS-PCR for the deletion of 16 nucleotides at target site two. To select for the deletion of 16 nucleotides, AS-primer MT-1.2 is used and for the corresponding wildtype allele primer WT-1.2. For all PCR reactions a common reverse primer is used (R-1) (C) Genotyping results using AS-primers for the deletion at target site two (MT-2) and the corresponding wildtype allele (WT-2). R-2 is used as reverse primer. (D) Expected

genotypes due to PCR results. Boxes with dotted lines symbolized the deletion site, boxes with solid lines the deletion.